

# Megazyme

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**ASSAY OF  
endo-PROTEASE  
using  
AZO-CASEIN**

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### **SUBSTRATE:**

Azo-Casein is prepared by dyeing casein with sulphanilic acid. The dyeing level is carefully controlled to produce a substrate which has about 5-times the sensitivity of similar products from other commercial suppliers (e.g Sigma Chemical Co., Azo-Casein Lot. 74H7165).

### **DISSOLUTION:**

To powdered substrate (2 grams) in a 120 ml beaker, is added 4 ml of ethanol or industrial methylated spirits (IMS). This is stirred on a magnetic stirrer to remove all "lumps", and is then treated with 96 ml of sodium phosphate buffer (100 mM, pH 7.0; Buffer A). The suspension is vigorously stirred on a magnetic stirrer until the substrate is completely dissolved (about 10 min). Any Azo-Casein which sticks to the edge of the beaker should be dislodged with a small spatula. The solution is stored in a well sealed glass Duran bottle, and is overlain with 2 drops of toluene to prevent microbial contamination. This solution is quite stable for several weeks when stored at 4°C.

### **APPLICATIONS:**

Azo-Casein can be used to assay all *endo*-protease enzymes which are active on casein. Such enzymes include bacterial alkaline proteases (e.g. Subtilisin A, as in Alcalase from Novo Nordisk), papain, trypsin, fungal and bacterial proteases and bromelain. In this booklet, standard curves are provided for Subtilisin A, trypsin and chymotrypsin. For several other proteases, regression equations are given. Assays employing Azo-Casein are less sensitive than those using Protazyme AK tablets (about 20-50% as sensitive, depending on the protease studied.)

Standard curves for a range of proteases have been prepared at pH 7.0. Since *endo*-proteases are used under a wide range of conditions of pH and temperature etc., it is not possible to generate curves for every situation. For specific needs, please contact Megazyme.

## BUFFERS FOR EXTRACTION, DILUTION AND ASSAY:

**BUFFER A:** (Sodium phosphate, 100 mM, pH 7)

17.8 g of di-sodium hydrogen phosphate dihydrate ( $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ ) is dissolved in 900 mL of distilled water and the pH is adjusted to 7.0 with 1 M HCl. The volume is adjusted to 1 litre. Store at 4°C. Sodium azide (0.2 g; Sigma S-2002) may be added as a preservative.

**BUFFER B:** (Sodium phosphate, 100 mM, pH 7),  
with cysteine (30 mM) and EDTA (30 mM)

8.9 g of di-sodium hydrogen phosphate dihydrate ( $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ ) is dissolved in 450 mL of distilled water and L-cysteine hydrochloride monohydrate (2.65 g; Sigma C-7880) and ethylenediaminetetra acetic acid (5.6 g, EDTA; Sigma ED2SS) are added and dissolved. The pH is adjusted to 7.0 with 1 M sodium hydroxide (40 g/L), and the volume is adjusted to 500 mL. Store at 4°C. Use within 2 days.

### NOTE

For the thiol-proteases (e.g. papain, bromelain and ficin) Buffer B is used for extraction and dilution. For other proteases, Buffer A is employed.

## ENZYME EXTRACTION and DILUTION:

Powdered enzyme preparation (1.0 g) is suspended in 50 mL of Buffer A or B and stirred on a magnetic stirrer for about 15 min at room temperature (until the powder is completely dissolved or dispersed). The preparation is then filtered or centrifuged (1,000g) if necessary. This **original extract** is then further diluted (1 mL to 9 mL of Buffer A or B) until a concentration suitable for assay is obtained.

Liquid enzyme preparation (1.0 mL) is added, using a positive displacement dispenser (e.g. Eppendorf Multipipette) to 49.0 mL of Buffer A or B and thoroughly mixed. The preparation is then filtered or centrifuged (1,000 g) if necessary. This **original extract** is then further diluted (1 mL to 9 mL of Buffer A or B) until a concentration suitable for assay is obtained (the dilution for Alcalase is 100-fold of the original extract).

## ASSAY PROCEDURE:

1. Pre-equilibrated enzyme solution (1.0 ml) [in buffer A or B (pH 7.0)] is added to pre-equilibrated substrate solution (1.0 ml) in Buffer A.
2. The solution is stirred on a vortex mixer and incubated at 40°C for 10 minutes.
3. The reaction is terminated and non-hydrolysed Azo-Casein is precipitated by the addition of 5% trichloroacetic acid (TCA, 6.0 ml) with vigorous stirring for 5 seconds on a vortex mixer.
4. The reaction tubes are allowed to equilibrate to room temperature for 5 minutes and then the contents are filtered through Whatman No.1 (9 cm) filter circles. Alternatively, the suspension can be centrifuged at 3,000 rpm (1,000g) for 10 minutes.
5. The absorbance of all filtrates (or supernatant solutions) are read against the reaction blank at 440 nm.

**Reaction blanks are prepared by adding the TCA to the substrate solution immediately before the enzyme preparation is added.**

## STANDARDISATION:

Standard curves relating the activity of Subtilisin A, trypsin and chymotrypsin on Azo-Casein (Lot 81001; absorbance 440 nm) at pH 7.0 and 40°C, to protease activity on casein (pH 7.0 and 40°C) are shown in Figures 1-3. It is apparent that the curve for subtilisin A is linear, whereas those for chymotrypsin and trypsin are not. The Regression equations for other proteases are also shown. The equations for subtilisin A, proteinase K and *A.niger* are very similar. The high slope values for papain and bromelain (in particular) may be due to the presence of high levels of peptidases in these preparations, which would be measured in the casein assay, but not in the Azo-Casein assay (which is specific for *endo*-protease activity). Further research on the latter proteases is underway.

### **PAPAIN (from *Papaya latex*):**

Protease (milli-Units/mL) = 270 × Abs. (440 nm) + 7; R = 0.99  
Linear absorbance range = 0.1 to 1.0

**BROMELAIN (from pineapple stem):**

Protease (milli-Units/mL) =  $460 \times \text{Abs. (440 nm)} - 13$ ; R = 0.99  
Linear absorbance range = 0.1 to 1.0

**FICIN (from figs):**

Protease (milli-Units/mL) =  $190 \times \text{Abs. (440 nm)} + 3$ ; R = 0.99  
Linear absorbance range = 0.1 to 1.1

**SUBTILISIN A (from *Bacillus licheniformis*):**

Protease (milli-Units/mL) =  $129 \times \text{Abs. (440 nm)} + 4$ ; R = 0.99  
Linear absorbance range = 0.1 to 1.0

**BACTERIAL PROTEASE (from *Bacillus subtilis*):**

Protease (milli-Units/mL) =  $250 \times \text{Abs. (440 nm)} - 8$ ; R = 0.99  
Linear absorbance range = 0.1 to 1.0

**PROTEINASE K (from *Tritirachium album*):**

Protease (milli-Units/mL) =  $140 \times \text{Abs. (440 nm)} - 4$ ; R = 0.99  
Linear absorbance range = 0.1 to 1.0

**FUNGAL PROTEASE (*A. niger*; from Sigma Chemical Co.):**

Protease (milli-Units/mL) =  $146 \times \text{Abs. (440 nm)} - 4$ ; R = 0.99  
Linear absorbance range = 0.1 to 1.0

**One Protease Unit is defined as the amount of enzyme required to hydrolyse (and TCA solubilise) one micromole of tyrosine equivalents per minute from soluble casein under standard assay conditions (pH 7.0 and 40°C).**

The reference protease assay uses casein as substrate, involves a TCA precipitation step and is standardised against tyrosine. This method is available on request.

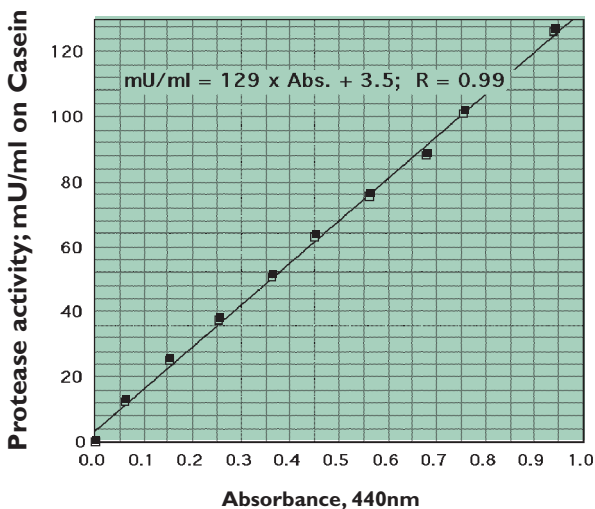


Figure 1. Subtilisin A Standard Curve on Azo-Casein (Lot 81001). Assay conditions; 10 min; pH 7.0; 40°C.

### CALCULATION OF ACTIVITY:

Protease activity is determined by reference to a standard curve (e.g. Figure 1) or to a Regression Equation to convert absorbance units to milliUnits of protease activity per assay (i.e. per 1.0 ml) and then calculated as follows:

### Units/ml of Original Preparation:

$$= \text{milliUnits/per assay} \times 50 \times \frac{1}{1000} \times \text{Dilution}$$

### where:

milliUnits per assay (i.e./1.0 ml) is obtained by reference to the standard curve or to the relevant Regression Equation.

50 = the volume of buffer used to extract the original preparation (i.e. 1g/50ml or 1ml of enzyme added to 49ml of buffer).

1/1000 = conversion from milliUnits to Units.

Dilution = dilution of the original enzyme preparation.

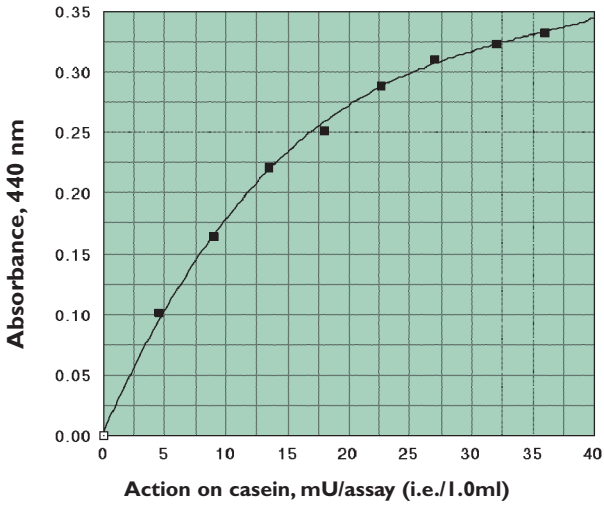


Figure 2. Trypsin Standard Curve on Azo-Casein (Lot 81001). Assay conditions; 10 min; pH 7.0; 40°C.

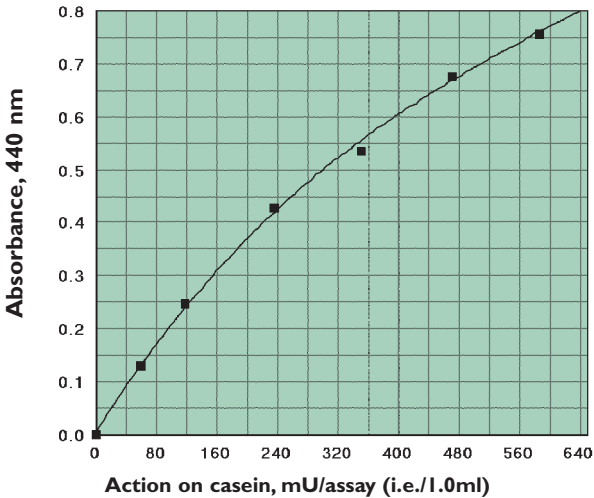


Figure 3. Chymotrypsin Standard Curve on Azo-Casein (Lot 81001). Assay conditions; 10 min; pH 7.0; 40°C.



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